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Cleavage of the cyclin-dependent kinase 5 activator p35 to p25 does not induce tau hyperphosphorylation

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Abstract

Hyperphosphorylated tau protein is the primary component of neurofibrillary tangles observed in several neurodegenerative disorders. It has been hypothesized that in certain pathological conditions, the calcium activated protease, calpain, would cleave the cyclin-dependent kinase 5 (cdk5) activator p35 to a p25 fragment, which would lead to augmented cdk5 activity, and cdk5-mediated tau hyperphosphorylation. To test this hypothesis, we induced calpain-mediated p35 cleavage in rat hippocampal neuronal cultures and studied the relationship between p25 production, cdk5 activity, and tau phosphorylation. In glutamate-treated cells p35 was cleaved to p25 and this was associated with elevated cdk5 activity. However, tau phosphorylation was concomitantly decreased at multiple sites. The calpain inhibitor MDL28170 prevented the cleavage of p35 but had no effect on tau phosphorylation, suggesting that calpain-mediated processes, i.e., the cleavage of p35 to p25 and cdk5 activation, do not contribute to tau phosphorylation in these conditions. Treatment of the neuronal cultures with *N*-methyl-D-aspartic acid or with calcium ionophores resulted in an outcome highly similar to that of glutamate. We conclude that, in neuronal cells, the cleavage of p35 to p25 is associated with increased activity of cdk5 but not with tau hyperphosphorylation.

Keywords: Alzheimer's disease; Cell death; Calpain; Glutamate; NMDA; Ionomycin; A23187; MDL28170

Tau is a microtubule-associated protein which regulates the assembly of tubulin into microtubules and also links microtubules with other cytoskeletal elements. The function of tau is regulated by phosphorylation in a complex manner where a number of phosphorylation sites, kinases, and phosphatases are involved. In several neurodegenerative disorders, including Alzheimer's disease (AD), abnormally hyperphosphorylated tau protein accumulates intracellularly. Several kinases have been suggested in the Alzheimer type of tau hyperphosphorylation. Cyclin-dependent kinase 5 (cdk5) has been implied as a major contributor in this process [1,2].

In neuronal cells, cdk5 phosphorylates several proteins involved in cytoskeletal dynamics, including tau [3,4]. The activity of cdk5 is regulated by binding of cdk5 activator proteins (p35, p39), as well as by phosphorylation. Recently it was observed that p35, the main activator of cdk5, can be cleaved to a p25 fragment by the calcium activated protease, calpain [5–7]. The p25 fragment has been reported to be a more potent activator of cdk5 and to have altered intracellular localization when compared to p35 [3]. However, the functional significance of p35 cleavage is still poorly understood.

In Alzheimer's disease, elevated calpain activity and p25 levels have been detected [3,8,9]. It has been hypothesized that in AD brain, perturbed cellular calcium homeostasis would induce calpain activation and cleavage of p35 to p25. This would result in dysregulated cdk5 activity and subsequent tau hyperphosphorylation. However, there is relatively limited evidence linking cleavage of p35 to p25 with tau hyperphosphorylation.

In this study, we examined the relationship between the calpain-mediated cleavage of p35, cdk5 activity, and tau phosphorylation in neuronal cultures. Glutamate treatment of the cultures was used to induce calpain activation

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and p35 cleavage. *N*-methyl-D-aspartic acid (NMDA) as well as the calcium ionophores ionomycin and A23187 were also employed. We examined the cleavage of p35 to p25 and compared this with cdk5 activity as well as with tau phosphorylation, assessed by Western blotting with several phosphorylation-dependent tau antibodies. The data were carefully related to markers of cellular integrity, such as cell morphology, lactate dehydrogenase (LDH) release, and cellular ATP levels.

Materials and methods

Neuronal cell culture. Primary cultures of rat hippocampal neurons were prepared and cultured as previously described [10]. After 13 days in culture the culture medium was supplemented with 2.5 mM $CaCl_2$ and the cells were subjected to experimental treatments. L-glutamate and NMDA were purchased from Sigma whereas A23187, ionomycin, and MDL28170 were from Calbiochem.

The cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer [20 mM Mops (3-[*N*-morpholino]propanesulfonic acid) (pH 7.2), 1 mM $MgCl_2$, 0.3 M NaCl, 0.5% Nonidet P-40, 50 mM NaF, 100 μM Na-orthovanadate, and 5 mM Na-pyrophosphate, 5 mM EDTA] containing complete protease inhibitor cocktail (Roche). The lysates were centrifuged at 13,000*g* (+4 °C) and supernatants were collected.

Western blotting. Western blotting was performed as previously described [10]. Primary antibodies were used at following dilutions: p35 (Santa Cruz, C-19), 1:600; cdk5 (Santa Cruz, C-8), 1:1000; tau-5 (BioSource, against phosphorylation-independent epitope of tau), 1:12,000; AT8 (Innogenetics, epitope around phosphorylated residue 202 of human tau), 1:3000; AT180 (Innogenetics, tau phosphorylated at residue 231), 1:2000; AT270 (Innogenetics, tau phosphorylated at residue 181), 1:12,000; pT212 (BioSource, tau phosphorylated at residue 212), 1:8000; pS404 (BioSource, tau phosphorylated at residue 404), and 1:8000; and αII-spectrin (Santa Cruz, C-20), 1:100.

Measurement of cdk5 activity. cdk5 activity was measured by a method modified from Saito et al. [11]. Fifty μg protein in 100 μl lysis buffer was incubated with 3 μl anti-cdk5 antibody (C-8) for 4 h at +4°C, 20 μl protein A–Sepharose suspension (Amersham) was added, and the incubation was continued overnight. The Sepharose was washed twice with washing buffer [10 mM Mops (pH 7.2), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM EDTA, 50 mM NaF, 100 μM Na-orthovanadate, and 5 mM Na-pyrophosphate] and once with assay buffer [10 mM Mops (pH 7.2), 1 mM $MgCl_2$]. Fifty μl assay buffer with 0.1 mM ATP, 0.4 mg/ml histone H1, and 0.1 mCi/ml [γ-^{32}P]ATP was added onto the Sepharose. The reaction was carried out for 30 min at 30 °C, and terminated by adding SDS–PAGE sample buffer and heating the samples for 5 min at 95 °C. The proteins were separated by SDS–PAGE, the gel was dried, radioactivity was detected by Storm PhosphorImager, and the pixel volume of the radioactive histone H1 band was quantified with ImageQuaNT software (Molecular Dynamics). Phosphorylation of histone H1 was totally abrogated by addition of 1 μM roscovitine, an inhibitor of cdk5, into the reaction.

Measurement of LDH release, ATP levels, and evaluation of neurite morphology. The extent of cell death was assessed by measuring the release of lactate dehydrogenase (LDH) into the culture medium with an enzymatic assay (Promega). Cellular damage was also characterized morphologically by evaluating neuritic changes with an inverted microscope. The neuritic network was visually graded to be either unchanged (−), or to have minor changes (+), moderate damage (++), or extensive damage (+++). For ATP measurement, the cells were washed with PBS, lysed in somatic cell ATP releasing reagent (Sigma), and the ATP levels were measured using a luciferase reaction based enzymatic assay (Sigma).

Results

Glutamate and NMDA induce p35 cleavage, cdk5 activation, and tau dephosphorylation

We treated rat hippocampal neuronal cultures with glutamate to examine the relationship between the cleavage of the cdk5 activator p35 to p25, cdk5 activity, and tau phosphorylation. In cultures exposed to glutamate, p35 was dose-dependently cleaved to p25 already after 30 min of treatment (Fig. 1A). The levels of cdk5 protein remained relatively constant until 2 h but decreased after a longer treatment (4–6 h). Cdk5 activity was elevated (up to 2-fold) in glutamate-treated cells in association with the increase in p25 after short exposure (30 min to 2 h), whereas no increase in cdk5 activity was detected after longer incubations (4–8 h).

Because cdk5 has been implied in tau hyperphosphorylation we examined tau phosphorylation in cells exposed to glutamate, using several tau antibodies. Western blotting with the phosphorylation-independent tau antibody, Tau-5, revealed two major tau bands (54 and 59 kDa) in untreated cells (Fig. 1A), whereas 200 μM glutamate induced an early (30 min to 1 h) transient appearance of two faster migrating tau bands (45 and 49 kDa), and loss of tau after a longer treatment (4–6 h). We hypothesized that the glutamate induced changes in the electrophoretic mobility of tau could result from dephosphorylation. Indeed, treatment of cell homogenates with λ-protein phosphatase resulted in similar changes in the electrophoretic mobility as detected after glutamate exposure (Fig. 1B), suggesting that the fast migrating bands in glutamate-treated cells represent dephosphorylated forms of tau. Tau phosphorylation was further assessed with five different antibodies that recognize phosphorylated forms of tau. Although cdk5 activity was increased in cells treated with glutamate, the amount of phosphorylated tau, detected by the antibodies AT180, pT212, AT8, pS404, and AT270, decreased to a varying extent in these cells (Fig. 1A). The decrease was detected already after 30 min of treatment with glutamate.

In general, the changes in p25 production, cdk5 activity, and tau phosphorylation in glutamate-treated cultures appeared before significant alterations in neurite morphology or loss of membrane integrity (LDH release) (Fig. 1A). However, the level of cellular ATP showed 20% or 45% decrease in cultures incubated for 1 h with 40 or 200 μM glutamate, respectively, indicating early disturbance of the energy metabolism. The changes in p25, cdk5 activity and tau detected after longer (4–8 h) incubations with glutamate were probably influenced by profound cell damage.

We also exposed neuronal cultures to glutamate in combination with MDL28170 (a calpain inhibitor) and found that MDL28170 prevented the cleavage of p35 to p25, as well as the cleavage of αII-spectrin, another

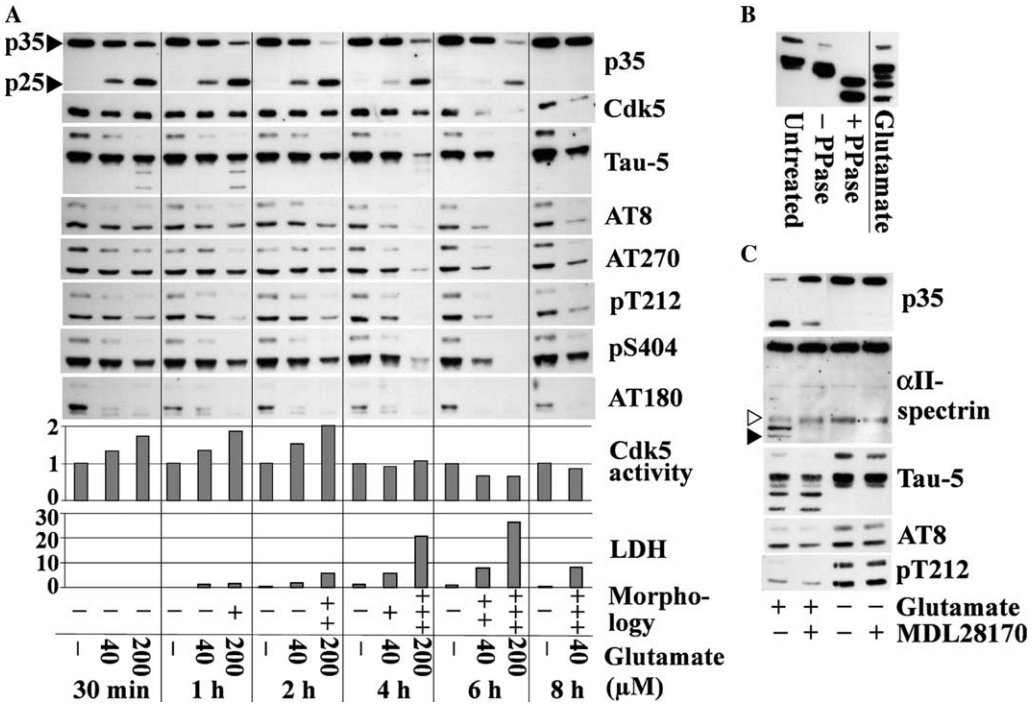

Fig. 1. Glutamate induces p35 cleavage to p25, increased cdk5 activity, and tau dephosphorylation in cultured neurons. (A) Rat hippocampal neuronal cultures were treated with L-glutamate and subjected to Western blotting as indicated in the figure. The neuritic network (morphology) was graded to be either unchanged (–), or to have minor changes (+), moderate damage (++), or extensive damage (+++). LDH release is presented as percentage of maximal LDH release induced by detergent lysis. The cdk5 activity values have been normalized to those of untreated control cultures of the corresponding time point (control = 1). (B) Neuronal cultures were either left untreated or subjected to 200 μM glutamate for 1 h. After cell lysis an aliquot of the untreated sample was diluted (1:14) in lysis buffer lacking NaF, Na-orthovanadate, Na-pyrophosphate, and EDTA but containing 1× $MnCl_2$ and 1× phosphatase buffer (New England BioLabs) and incubated for 1 h at 30 °C in the presence or absence of λ-protein phosphatase (New England BioLabs). The samples were analyzed by Western blotting with Tau-5 antibody. (C) Neuronal cultures were pretreated for 15 min with the calpain inhibitor MDL28170 and thereafter subjected to glutamate (200 μM) for 1 h and analyzed by Western blotting. αII-Spectrin antibody revealed a characteristic doublet of cleavage products in glutamate-treated cells (arrowhead). An unspecific band is pointed out by an open arrowhead.

calpain substrate, demonstrating the involvement of calpain in p35 cleavage (Fig. 1C). However, MDL28170 had no effect on tau phosphorylation in glutamate-treated cells, suggesting that calpain-mediated processes, in particular the cleavage of p35 to p25, do not contribute to tau phosphorylation in these conditions.

We then examined the effects of NMDA, an agonist of the NMDA-type glutamate receptor, in neuronal cell cultures. NMDA treatment resulted in similar changes as detected with glutamate. Thus, a rapid partial cleavage of p35 to p25, decreased tau phosphorylation, and loss of tau at longer incubations (6 h) were observed (Fig. 2). These changes appeared well before significant changes in neuritic morphology or LDH release (Fig. 2), whereas the amounts of cellular ATP were decreased by 19% and 25% with 80 and 400 μM NMDA, respectively, when compared to an untreated culture after 1 h of incubation.

We also tested the effects of a short transient exposure (15 min) to glutamate and NMDA. In these experiments, the levels of p35/p25, cdk5 activity, and tau phosphorylation were measured during a recovery period of up to 6 h. These conditions resulted in transient increases in p25 level and cdk5 activity that lasted up to 3 h after the

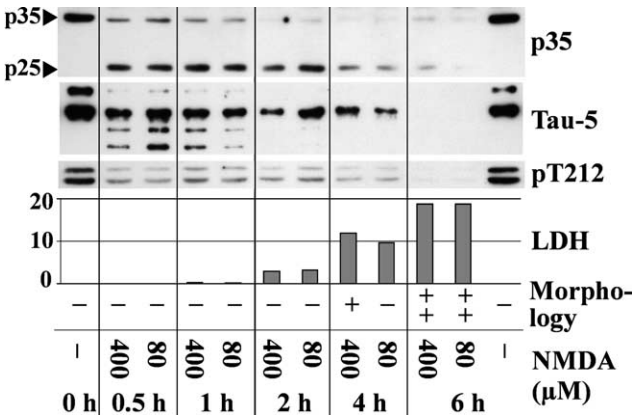

Fig. 2. NMDA induces p35 cleavage to p25 and tau dephosphorylation. Neuronal cultures were treated with NMDA and subjected to protein analysis by Western blotting. The neuritic network (morphology) was graded to be either unchanged (–), or to have minor changes (+), moderate damage (++), or extensive damage (+++). LDH release is presented as percentage of maximal LDH release induced by detergent lysis.

treatment. Tau phosphorylation was significantly decreased after 15 min, whereas no changes in phosphorylation were found after 1–3 h (data not shown).

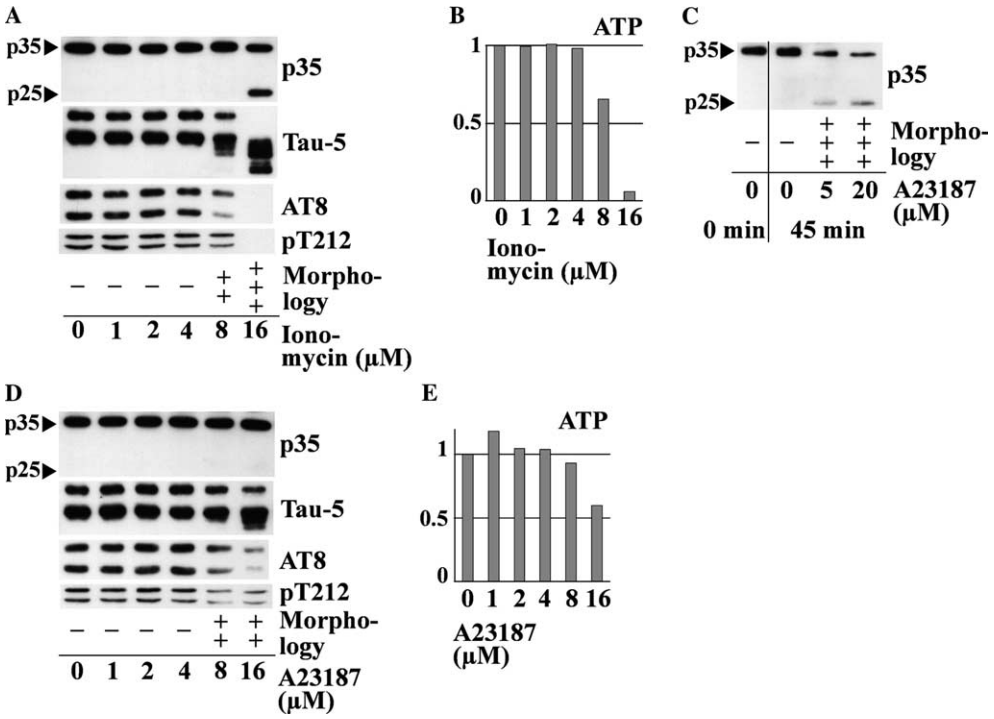

Fig. 3. Calcium ionophores result in cleavage of p35 to p25, tau dephosphorylation, and reduced ATP levels in neuronal cultures. Neuronal cultures were treated with ionomycin (A, B) or A23187 (C–E). Incubation time with the drugs was 40 min. In (C), a 45-min incubation time and 8-day-old cultures were used. The cell lysates were subjected to protein analysis by Western blotting (A, C, D) or measurement of ATP levels (B, E). The neuritic network (morphology) was graded to be either unchanged (−), or to have minor changes (+), moderate damage (++), or extensive damage (+++). Cellular ATP levels were normalized to that observed in untreated culture (untreated = 1).

Calcium ionophores induce p35 cleavage and tau dephosphorylation

Rat hippocampal neurons treated with calcium ionophores ionomycin and A23187 showed an increase in p35 cleavage to p25 (Figs. 3A and C). We also detected a decrease of tau phosphorylation in these cells (Figs. 3A and D), consistent with the results with glutamate and NMDA. Calcium ionophores changed p25 level and tau phosphorylation concomitantly with morphological alterations, thus differing from glutamate and NMDA treatments in which the changes in p25 level and tau phosphorylation appeared before morphological evidence of cell damage. However, similar to glutamate and NMDA treatments also the calcium ionophores caused an early decrease in ATP levels, temporally paralleling the changes in p25 and tau phosphorylation (Figs. 3B and E). In cells subjected to the calcium ionophores, the decrease in tau phosphorylation appeared at ionophore concentrations that did not increase p25 (Figs. 3A and D).

Discussion

Hyperphosphorylation of tau occurs during the formation of neurofibrillary tangles observed in several neurodegenerative disorders. It has been hypothesized that calpain-mediated cleavage of the cdk5 activator p35 to p25 would lead to augmented cdk5 activity and subsequent tau hyperphosphorylation. To test this hypothesis, we treated rat hippocampal neuronal cultures with glutamate and NMDA as well as with the calcium ionophores A23187 and ionomycin, and studied the relationship between p25 production, cdk5 activity, and tau phosphorylation. In cells treated with glutamate or NMDA, the increased production of p25 was associated with a moderate increase in cdk5 activity. However, tau phosphorylation was concomitantly decreased at multiple sites. These changes occurred well before cell death-associated changes such as neurite damage or LDH release, although cellular ATP levels were concomitantly reduced. The calcium ionophores also induced p35 cleavage to p25, as well as reduced phosphorylation of tau.

There has recently been considerable interest in the calpain-mediated proteolytical cleavage of the cdk5 activator p35, and on the functional significance of this phenomenon in relation to different pathological conditions. Cleavage of p35 has been detected in cell cultures challenged with substances like glutamate, H_2O_2, β-amyloid, staurosporine, maitotoxin, cyclophosphamide, and calcium ionophores [5–7,12,13]. Moreover, an increase of p25 is also observed in animal models of ischemia [5,7] and amyotrophic lateral

sclerosis [14]. Several studies have suggested a linkage between p35 cleavage to p25 and increased cdk5 activity [3,12–14]. Our current data confirm that in neuronal cultures p35 is cleaved to p25, resulting in elevated cdk5 activity in conditions that involve calcium influx and calpain activation. Previous studies have not revealed whether p35 cleavage is an early or late event in calcium-induced toxicity. Our results show that in glutamate- and NMDA-treated cells p25 is produced before significant morphological alterations or LDH release. However, cellular energy metabolism appeared to be compromised concomitantly with p25 production, as suggested by the decrease in cellular ATP levels.

Previous studies on the role of p35 cleavage induced cdk5 activation in tau phosphorylation have produced controversial results, possibly due to differences in experimental approaches. Our data show that in neuronal cells, tau phosphorylation decreased at multiple sites during calcium-mediated toxicity, although the endogenous p35 was cleaved to p25 and cdk5 activity was elevated. Thus, the possible minor effects of the elevated cdk5 activity on tau phosphorylation could have been overridden by other concomitant changes occurring in the cells during the toxic insults. However, calpain inhibition in glutamate-treated cells had no obvious effect on tau phosphorylation, further implying that calpain-mediated processes, including cleavage of p35 and cdk5 activation, do not contribute to tau phosphorylation in these conditions. Previously, Patrick et al. [3] suggested that an increase of p25 level may lead to augmented tau phosphorylation, because cells overexpressing p25 displayed increased phosphorylation of tau as compared to cells producing p35. Town et al. [12] observed elevated tau phosphorylation concomitantly with p25 production, when a p35-transfected cell line was treated with β-amyloid. Also, increased phosphorylation of tau in association with p25 production was detected in malonate injected rat brains [7]. Transgenic mice expressing p25 have shown cytoskeletal and axonal abnormalities as well as elevated tau phosphorylation [15,16], although the possible differential effects of p35 and p25 overexpression have not been investigated in these animals. In another study overexpression of p25 resulted in unaltered tau phosphorylation in mice [17]. Furthermore, treatment of neuronal cultures with the calcium ionophore A23187 has been shown to induce p35 cleavage, accompanied by a decrease in tau phosphorylation [17].

Our results showing tau dephosphorylation in neuronal cultures treated with glutamate or NMDA are in agreement with some previous studies [18–21], whereas other studies have suggested increased phosphorylation of tau [22,23]. Likewise, calcium ionophores have previously been shown to induce both dephosphorylation [17,21,24] and hyperphosphorylation of tau [25]. Dephosphorylation of tau observed in our study may result from the activation of calcineurin (protein phosphatase 2B), a calcium-dependent protein phosphatase, in these conditions [20,24]. On the other hand, previous studies have shown that glutamate toxicity causes a transient inhibition of mitochondrial function and decreased ATP synthesis [26]. It is therefore possible that shortage of ATP, a substrate of kinase reactions, could directly affect the phosphorylation level of tau. Accordingly, our data showed that decreased tau phosphorylation appeared in association with a reduction of cellular ATP. However, in a study by Norman and Johnson [27], depletion of ATP was not sufficient to induce dephosphorylation of tau.

In conclusion, our data showed that calpain-mediated cleavage of the cdk5 activator p35 to p25 is associated with increased cdk5 activity but not with tau hyperphosphorylation in neuronal cell culture models of calcium-mediated toxicity. Thus, our results do not support the hypothesis that p35 cleavage to p25 invariably results in tau hyperphosphorylation. However, our results do not refute the possible contribution of cdk5 to tau hyperphosphorylation in general. Also, the calpain-mediated p35 cleavage could lead to increased phosphorylation of cdk5 substrates other than tau.

Acknowledgments

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